IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 3, Issue 4 (Jul. – Aug. 2017), PP 08-14
www.iosrjournals.org

Biogas Production From Organic Wastes Of Paper And Leather Industries

Isabella Apruzzese[1], Vibha Bhardwaj[1], Giuliano Degrassi[1],
Rakesh Kumar Bhardwaj[*2]
[1] Industrial Biotechnology Group, International Center for Genetic Engineering and Biotechnology, IBioBA-ICGEB, Godoy Cruz 2390, Buenos Aires, Argentina.
*[*2] BLJS Post Graduate College Tosham -127040 INDIA.*
** corresponding author: Rakesh Kumar Bhardwaj*

Abstract: *Several industrial processes exist that convert raw agricultural products in goods through mechanical and chemical treatments. In some of these processes the adoption of biotechnology-based production steps can be considered for optimization of performances and reduction of the environmental impact. One example of biotechnological approach developed and adopted in the pulp and paper industry is bioleaching, the removal of hemicelluloses from cellulose by means of enzymatic treatment. This treatment has the advantage of reducing the use of chlorine and the related environmental impact. However if we consider the entire industrial process, there is another major step that could benefit from the potential of biotechnology, the disposal of waste generated by the transformation of wood into paper. Similar problems exist also in the leather industry where the generated waste contains organic and inorganic components that make their disposal expensive and environmental risky. In this review we analyze the state of the art of these two industrial activities in relation to the possibility of developing biotechnological products and processes for the conversion of wastes into energy, with the aim of reducing the cost of production and the environmental impact, and generating two value-added products through anaerobic fermentation: biogas and organic fertilizer.*

Key words: *Paper and pulp industry, Bioleaching, Environment impact, Tannery, Cellulases*

Date of Submision : 17-08-2017 Date of acceptance : 28-08-2017

I. Introduction

Among the many industries that transform raw materials and derivatives of agricultural and livestock origin for production of durable goods and intermediate goods for making of other products, paper and pulp factories and leather industries are interesting because they offer the possibility of improvement by means of adopting biotechnological steps aimed at improving the process efficiency and reducing the environmental impact. In fact, through a process called “anaerobic digestion”, these industrial procedures permit the use of their own wastes to obtain biogas and fertilizers. A method like this increases the whole process’s efficiency and considerably affects in a positive way its environmental impact and economic sustainability. These are indeed one of the main focuses of nowadays European and global politics, and they represent a common goal in order to safeguard the planet. Biotechnologies already play a role in the environmental sustainability of the manufacturing of the main products of these industries: e.g., the traditional process of bleaching has a greener alternative called biobleaching, which can reduce or eliminate the use of chlorine-based chemicals that would produce toxic and non-biodegradable pollutants thanks to enzymes. However this is just a small part of the whole production system. Waste disposal represents a considerable cost in terms of pollution and economy, and there is an urgent need to find a sustainable way to use scrap or to eliminate it.

Solid wastes coming from pulp and paper factories and from leather industry can be used to both produce biogas and bio-fertilizers: this process allows optimizing the investments and improving the process’s efficiency and sustainability. This procedure depends on anaerobic digestion, which allows the conversion of complex organic matter into methane and carbon dioxide. This process consists of many steps, but the rate limiting one is the hydrolysis of complex polymers into simple compounds. Hence, making the hydrolysis reaction faster using biotechnological tools would significantly enhance this rate limiting step and improve the efficiency of the whole process, making it competitive on the market: in one step wastes would be disposed, a new source of energy would be created and potential material that could be used as a fertilizer would be made. Therefore there is a strong interest in pretreatment methods of complex polymers. Here we focus on the biotechnological tools; however physical and chemical technologies have also been developed (Elliott & Mahmood, 2007). Different approaches have been studied, that include the use of recombinant enzymes or the study of cellulosomes. In this mini review, we suggest the production of recombinant organisms as the method of choice to express the cocktail of enzymes necessary to complete the hydrolysis process, with an eye on *Pichia pastoris*, whose advantages in terms of large scale productions are well known: high yields and high secretion levels, which make it easier to purify the proteins.

II. Pulp, Paper And Leather Industry: Importance, Production And Waste

2.1 Pulp and paper industry- Pulp and paper industry is one of the largest industries in the world: the total paper production amounted to 403 million tons in 2013, and it has been estimated that global paper consumption in 2025 will amount to 500 million tons. Asia is by far the world's biggest producer and consumer of paper, followed by Europe and North America (Bajpai, 2015). One possible explored utilization of non-edible biomass is its conversion into glucose or other fermentable simple sugars, and the following fermentation of the sugar into final bioproducts. Among various biomass types, wastes containing cellulose and other plant cell wall components are particularly suitable for bioconversion into valuable bioproducts.

An advanced technology has been proposed for complete utilization of these wastes by bioconversion. The technology is comprised of the following main steps: (1) re-dispersion of the waste and then screening of the pulp in order to separate industrial organic waste (IOW) from mineral residues; (2) acidification and washing of the IOW to remove the residual mineral residues; (3) high-solids enzymatic hydrolysis of the demineralized residues to obtain fermentable sugars; and (4) fermentation of the sugars into biofuel. If the waste is constituted by cellulose fiber, from 1 ton of waste paper and cellulose about 280 liters of bioethanol can be produced that have capacity of the generating energy up to 1680 kWh. Besides, remaining by-products of the processing include about 260 kg of residual fibers and about 270kg of mineral fillers that can be used repeatedly in the papermaking (Reena 2016). This is particularly true in those paper industries using pulp instead of wood and generating wastes containing short fiber cellulose, disposed because unable to make paper: this cellulose-containing waste could be utilized completely for production of the valuable bio products.

Pulp and paper industry converts wood or recycled fiber into pulp and primary forms of paper. First mechanical and then chemical methods have been developed to produce pulp from wood. Pulp mills separate fibers of wood or from other materials such as rags, wastepaper or straw, in order to create pulp. Paper mills primarily are engaged in manufacturing paper from wood pulp and other fiber pulp and may also manufacture converted paper products. Therefore, pulp and paper industry is a major consumer of natural resources (wood), energy (fossil fuels but with a substantial share of bioenergy, electricity), water, and it is a significant contributor of pollutant discharges and emission to the environment. Lately, the main goal has been to minimize the impact that it has on the environment by reducing to minimum the consumption of resources and emissions and by minimizing cross-media effects. To do this, all the elements of production must be taken into account.

Concerning the emission to water, public concern is about the potential environmental hazard that comes from the use of chlorine in the bleach plants. In the last years this has been reduced and replaced: the reduction of chlorinated organics in the effluents of pulp mills was made possible thanks to measures such as increased delignification before the bleach plant and the installation of external treatment plants of different designs. Notwithstanding the considerable improvements made in managing water emissions, the reduction of the remaining load of poorly biodegradable organic substances, the emission of nutrients and the discharge of suspended solid will continue to be a challenge for pulp and paper industry in the future. Furthermore, in the past years sulphur air emissions have been considerably reduced by substantial growth in technology, such as high efficiency combustion and flue-gas cleaning. However, the need for heat and power causes significant industrial emissions to air.

The situation is significantly different for solid wastes, since the goal for the management of residues includes their use as source of renewable energy, soil fertilizer or raw material for other industries; in addition the conversion into added value products for other users is also an option. In general the target is to minimize the amount of waste to be sent to landfills (Suhr, et al., 2015).

2.2 **Leather Industry**

Leather industry has a significant economic influence as well. FAO's data indicate that about 1.67 x 10^9 m^2 of leather are made annually in the world, and it is estimated to be a 70 billion dollars annual global trade (Masciana, 2015). Among the different processes to produce durable and flexible leather there is tanning of putrescible animal rawhide and skin, which can be done in a variety of ways depending on the form of leather. This is a process that converts raw hide of skin into a stable material that dries out to a flexible form without putrefying and becomes suitable for a wide variety of end applications. During this process, an enormous amount of water and pollutants are discharged: the variation of pH leads to an increase in Chemical Oxygen Demand (COD), total dissolved solids, chlorides, sulphates in tannery, wastewaters. This gives unfavorable consequences on environment but also affects the efficiency of effluent treatment plants. Tanned leather is non-biodegradable because a great deal sludge is generated from the tannery plants. Pollutants such as ammonia, hydrogen sulfide, volatile hydrocarbons, aldehydes are emitted in the atmosphere from tannery plants as effluents. Finally, a wide variety of chemicals are used in order to bring leather to its usable form. There are restrictions on their use, mainly due to high persistence of the chemicals in the environment, because of their low bio-degradability. To reduce negative environmental impact there are two broad methods: low waste or cleaner technologies that avoid the use of harmful chemicals and produce solid wastes that can be used as by-products, and treatment of wastewater and environment-friendly handling and processing of solid waste (Dixit, et al., 2015). Therefore, leather industry requires three main production steps, i.e. pre-tanning, tanning, post-tanning. At each step various chemicals are used and a variety of materials are expelled with a total amount of 30-40 x 10^{10} litres of liquid effluents generated. This leads to two main problems: availability of good quality water and the treatment of effluents. It is reported that 70% of pollution originates from pre-tanning operations (Ramasami, 1998).

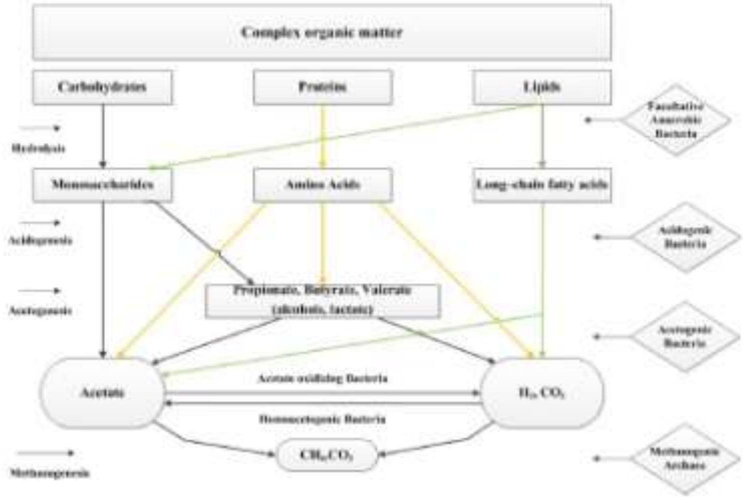
III. Biogas alternative

The increasing demand for energy has led to the accumulation of greenhouse gases, which are mainly composed by carbon dioxide obtained from the combustion of fossil fuels. It has been demonstrated that this phenomenon will lead to climatic changes such as an increase in surface air temperature of approximately 5°C until 2100. Hence, it is becoming more and more necessary the identification and development of strategies to face this problem: these come from economy, politics, but also from scientists, engineers and venture capitalists. Moreover an improvement in energy is needed, which means the use of less carbon-intensive fuels and renewable energy resources.

A suitable option is biogas, which is mainly composed of methane and carbon dioxide. The term generally refers to a gas produced by breaking down the organic matter without oxygen. Organic wastes may be converted into biogas, which is produced by anaerobic digestion or fermentation of biodegradable materials (Kamali, et al., 2016). Therefore methane, which is a non CO_2 greenhouse gas with a high greenhouse warming potential can be used as a hydrocarbon molecule: it has a low emission factor and a renewable origin. Hence, its use contributes to the reduction of methane emission naturally occuring in anaerobic degradation of organic matter. This is particularly important for industrial organic wastes (IOW) because they have an impact on the environment and represent a cost due to the need of proper disposal. In order to improve biogas purity and reduce pipeline corrosion induced by carbonic acid, the separation of CO_2 from methane is necessary (Appels, et al., 2011).

IV. Waste-to-energy conversion is made possible through anaerobic digestion

There is a large-scale contamination of land, water and air resulting from unmanaged organic fractions from farming, industry and municipalities that decompose in the environment. A solution to reduce bio-degradable organic wastes might be anaerobic digestion, a suitable waste-to-energy technology to treat solid waste and waste water with consequent enhancement of environmental quality and suitable energy production. Anaerobic digestion is a process in which a complex mix of microorganisms transforms organic materials under oxygen-free conditions into biogas, nutrients and additional matter. Raw biogas typically consists of methane (60%) and carbon dioxide (40%), as well as water vapor and trace amounts of hydrogen sulfide. Besides, anaerobic digestion stabilizes the organic matter in wastewater solids and reduces pathogens and odors. The reduction of total solids by converting part of the volatile solids fraction into biogas is a process composed by several steps:



(from Merlin Christy et al., 2014)

1) **Hydrolysis:** depolymerisation of organic matter from insoluble substrate (polysaccharides) to smaller units through hydrolytic microorganisms secreting different hydrolyzing enzymes such as cellulases, amylases, cellobiases, xylanases, proteases, lipases. First, the bacteria on the particle surface release enzymes and produce monomers which can be used by hydrolyzing bacteria themselves; second, degradation of particle surface by bacteria occurs. Considering $C_6H_{12}O_4$ as an approximate formula for the mixture of organic waste, a general hydrolysis of polysaccharides reaction might be:

$$(C_6H_{12}O_4)_n + nH_2O \rightarrow nC_6H_{12}O_6 + n2H_2$$

In this step, polymeric matter is transformed into monomers: lipids are converted into fatty acids, polysaccharides into monosaccharides, proteins into amino acids and nucleic acids into purines and pyrimidines. For these reactions several different enzymes secreted by microbes are needed.

Accessibility of hydrolytic microorganisms to the solid matter and anaerobic digestion of solid lignocellulosic material is the rate limiting step of the process. Hence, the solid matter is pre-treated in order to break the polymer;

2) **Acidogenesis:** amino acids and fatty acids are used as substrate for fermentative microorganisms to produce organic acids such as acetic, propionic and butyric acids, ethanol, propionate (volatile fatty acids, VFA) which cause a drop in pH due to an increase in hydrogen concentration. This condition is beneficial for acidogenic and acetogenic bacteria;
3) **Acetogenesis:** organic acids are degraded by the obligated hydrogen producing acetogenic bacteria to give carbon dioxide and acetic acid. These microorganisms are strict anaerobes, have optimum pH around 6 and they are slow growing and sensitive to environmental changes.
4) **Methanogenesis:** Methanogenic microorganisms that belong to Archea produce methane as a metabolic byproduct, starting from H_2/CO_2, formate, methylated C1 compounds or acetate. The reaction occurs either by cleavage of acetic acid molecules to generate carbon dioxide and methane or by reduction of carbon dioxide with hydrogen. The first method uses methyl-coenzime M reductase and is the most common way to obtain methane: bacteria that use hydrogen are more resistant to environmental changes and slightly prefer alkaline environment. Methanogenesis is the rate controlling portion of the anaerobic process (Merlin Christy, et al., 2014).

V. Use Of Digestate As A Fertilizer

Organic waste produced by agroindustries is a rich source of nutrients that might be exploited in agriculture to recycle nutrients and to reduce waste discharge and chemical fertilizers. However this waste should undergo several modifications in order to be used successfully without negative effects. Thus, anaerobic digestion can be used not only as a waste-to-energy conversion technology, but also to allow nutrients to remain in the digested material. The by-product deriving from the conversion of biomass into biogas is known as digestate. It contains partially degraded organic matter, residues of the microflora developed during the biogas production, inorganic compounds and, very important for the fertilization point of view, nitrogen, phosphorous and potassium in different concentrations ranging from 2.3 to 4.2, from 0.2 to 1.5 and from 1.3 to 5.2 kg/t, respectively (Alburquerque, et al., 2012). The use of waste-derived materials for agricultural purposes is risky unless the material properties are carefully evaluated. Ammonium, for example, is generated from mature proteins and this can be considered a nuisance because ammonium might convert into ammonia gas and be toxic. Thus, positive effects can be expected if the digestates are applied directly with incorporation into the soil immediately after spreading. In addition, significant differences were found when the solid and the liquid fractions of the digestate were evaluated as fertilizers, with the solid one being more suitable for this use and less toxic than the liquid one (Stefaniuk, et al., 2015).

VI. Pre-Treatment Of Paper And Cardboard Waste To Improve Biogas Yield

Diffraction analysis has shown different crystalline forms of cellulose, which is highly resistant to microbial and enzymatic degradation. In contrast, amorphous cellulose is degraded faster and easier: the degree of crystallinity affects enzymatic hydrolysis of cellulose and is the major obstacle to its utilization to produce fermentable sugar economically (Kumar, et al., 2009). A variety of pretreatment procedures have been evaluated for their effectiveness towards the degradation of lignocellulosic materials. There have been studies on the improvement of biogas production after pretreatment with cellulases and cellulase-producing microorganisms. However very few pretreatment methods have been effective for increasing biogas production of waste paper and cardboard (Cater, et al., 2014).

Pretreatment of biomass feedstocks, which are rich in cellulose and lignin, can break down recalcitrant polymers and increase biogas production and volatile solids reduction and increased solubilization. Pretreatment can be done with physical, thermal, chemical methods or a combination of them. Increased gas production and reduced excess sludge have been reported to be the added benefits associated with them. In particular, the technologies that appear to be at the forefront of preconditioning sludge include systems based on sonication, thermal processing and mechanical disintegration (Damm, et al., 2016).

Biological pretreatment offers some important advantages, such as low chemical and energy use. Microorganisms like brown-, white- and soft-rot fungi are used to degrade lignin and hemicellulose in waste materials. Lignin degradation by white-rot fungi occurs through the action of lignin degrading enzymes such as peroxidases and laccases. These enzymes are regulated by carbon and nitrogen sources (Kumar, et al., 2008).

The C/N ratio is important for biomass pretreatment, because degradation of lignocellulosic material depends on the material C/N ratio. To degrade each molecule of carbon, a definite proportion of nitrogen is required by the microorganisms, and this varies with different kinds of microflora. Fungi have higher C/N ratios compared to bacteria; therefore, fungi are more capable of degrading lignocellulose (Mao, et al., 2015). Yuan and Cui (2012) have studied the effect of pretreatment by a microbial consortium on methane production of waste paper and cardboard. The results they obtained demonstrated that sCOD concentration reaches a peak at day 7 of pretreatment; pH decreased to its lowest value between day 4 and day 7, showing a change typical of lignocellulose degradation by the studied consortium; the degradation of the four substrates mainly occurred during the first 7 days; a quantitative analysis of major volatile organic products showed that their concentration increased at the early stages of pretreatment and then decreased gradually (this last trend indicating that the production of VFA is the direct cause of pH evolution and is influenced by the lignin content). In general Yuan and Cui (2012) showed that the optimal length of time for pretreatment of waste paper and cardboard is 7 days.

VII. Heterologous Expression Of Lignocellulolytic And Keratinolytic Enzymes

Conversion of cellulosic substrates into first precursor products requires the synergistic action of endo/exo cellulases and beta-glucosidases, and microbial strains with cellulolytic activity usually show limiting levels for one of the enzymatic components. Hence, attempts have been made to increase the levels of these components through genetic manipulation: genes for cellulolytic enzymes were cloned and expressed in microorganisms to improve bioethanol and biogas production (Chandel, et al., 2012).

Recombinant DNA technology offers significant potential for improving various aspects of lignocellulolytic enzymes such as production, specific activity, pH and temperature stability.

Hence, heterologous expression can be considered as a powerful tool to improve production yield and activity of enzymes: it gives the possibility to obtain robust strains that can express specific cellulases with specific activities that are based on the need of a specific system. Desired enzymes are obtained with the desired outcomes (Dashtban, et al., 2009).

The fact that large amounts of enzymes are required for the enzymatic conversion of cellulose to fermentable sugars impacts on the cost-effectiveness of the process. A one-step conversion with one single organism, capable of cellulose degradation and efficient fermentation would greatly enhance cost effectiveness. This kind of organisms are not currently available, therefore, one strategy is to construct suitable organisms that exhibit high product yields to produce a heterologous cellulose utilization (Garvey, et al., 2013). Two major problems to be solved when these enzymes are heterologously expressed in high amount are (i) the possible lack of solubility in the heterologous expression system (Baneyx & Mujacic, 2004), and (ii) the need to have them secreted into the medium in order to be able to recover only the cell-free supernatant and overcome the regulatory constraints of the use of a genetically modified microorganism. One heterologous expression system that has been successfully used to achieve these goals is *Pichia pastoris* (Chatani et al., 2000).

Indeed, the newly synthesized recombinant protein is expressed in a microenvironment that differs from the one of the original source. Moreover, high levels of expression may lead to interactions of hydrophobic stretches present at high concentrations in the polypeptide: this causes protein instability and aggregation. Inclusion bodies formation results from an unbalanced equilibrium between protein aggregation and solubilization. However, inclusion bodies formation can be an advantage when the protein can be easily refolded *in vitro*: in this case conditions may be adjusted to favor the formation of inclusion bodies, providing a simple method for achieving a significant 1-step purification of the expressed protein (Rosano & Ceccarelli, 2014).

Many proteins that form inactive inclusion bodies in prokaryotic systems such as *E. coli*, are expressed in their biologically active state in *P. pastoris*. This eukaryotic system has many advantages: first of all, its respiratory growth enables it to be cultured to high cell densities; second, it is capable of post-translational modifications (i.e., proteolytic processing, glycosylation, disulphide bridge formation). It is cost-effective and less time-consuming: it is easy to scale-up and easy to handle. It brings to high protein yields and high levels of secretion (Kalidas, 1999).

A conceptual comparison can be made between cellulose and keratin: the majority of industrial biotechnology mainly focus on the conversion of plant masses materials (therefore lignocellulosic materials). However, animal-derived biomass is gradually attracting more attention because keratinaceous waste stream provides an underexploited source of animal feed proteins. A greater exploitation would achieve increased and improved resource efficiency and benefits for the environment (Thanikaivelan, et al., 2004). Similarly to cellulolytic enzymes, keratin degrading proteases act together with other keratinolytic enzymes to decompose keratin. Therefore, there are enzymatic and biochemical mechanisms that act synergistically also for keratin decomposition (Lange, et al., 2016). Isolation and cloning of keratinase genes are essential to ensure improved enzyme yields to allow commercialization. A few attempts have been made to isolate and clone keratinase genes from bacteria and actinomycete, however, in most of these expression studies it has been observed that plasmid-based expression in *E. coli* or *B. subtilis* are unstable (Gupta & Ramnani, 2006).

7.1 Heterologous expression: state of the art

The main goal for recombinant strategies to produce cellulolytic enzymes is the so-called "consolidated bioprocessing", which combines hydrolysis of cellulose and fermentation, which means expressing microbial cellulases in non-cellulolytic systems that display high-product yields.

Several expression systems have been studied.

There are several main advantages in the use of yeast expression systems: they are simple to handle and require inexpensive media formulations. Plus, they rapidly reach high-cell densities and they do not show viral inclusion or presence of pathogens. However, they have a limited secretion capacity.

The most common yeasts used to heterologously express proteins are *Saccharomyces cerevisiae*, *Pichia pastoris* and *Kluyveromyces marxianus*. On the other hand, bacterial expression systems are easy to manipulate, and show increases in enzyme yields compared to the ones of the original hosts. The enzymes obtained are easy to express and purify. Recombinant cellulases were obtained in *Geobacillus*, which showed cellulolytic activity under extreme industrial processing conditions. Thermophilic bacteria were also obtained but they demonstrated lower amounts of enzymes compared to mesophiles.

In general, bacterial expression systems have several ongoing problems: truncation of enzyme may occur due to the lack of glycosylation of cellulases of fungal origins, and so does codon mismatching. Also plant expression systems were studied: they have low production costs and they are easy to scale up. However, the overproduction of recombinant cellulases might negatively influence plant growth and development, thus affecting the total yield of product. Lastly, there are several studies concerning the use of mixed culture systems, that is, the use of complex microbial communities for the bioconversion of biomass. These systems increase the growth and activity of cellulolytic bacteria under unfavorable environmental conditions. Wongwilaiwalin and co-workers (2010) used a microbial consortium with different groups of microbes. Results revealed that ligno-cellulolytic enzyme systems are suitable for biomass degradation and conversion in biotechnological industry (Behera & Ray, 2016).

VIII. Conclusions and perspectives

The complexity of waste-to-energy conversion processes should be converted into what is called "Consolidated Bioprocessing", through which production costs would be considerably reduced. This goal can be achieved using biotechnologies, a powerful and promising tool that allows to genetically manipulate microorganisms.

In this minireview, the focus goes to heterologous expression of lignocellulose and keratine degrading enzymes. Both these polymers need to be hydrolyzed by a cocktail of enzymes, rather than one single enzyme, to be converted in the correspondent monomers which will then be used for anaerobic digestion. Therefore, it is necessary to find a suitable expression system in order to express these enzymes through one single microorganism. Here, we suggest the use of *Pichia pastoris*, which has demonstrated to have a lot of advantages over other expression systems. Prokaryotic systems such as *E. coli* have resulted to be inefficient despite their unquestionable advantages on yields and economics: strategies and protocols have been studied in order to obtain heterologously expressed proteins which were also soluble and functional (Pancheco, et al., 2012). Instead, *Pichia pastoris* can produce post translational modifications and secrete proteins at high yields, it is easy to manipulate and it is cost-effective. Hence, it is provided with all the industrially relevant features.

The goal now is to heterologously express a cocktail of enzymes which is also compatible with industrial needs, in order to develop anaerobic digestion in a single bioconverter.

Until now, the use of yeast systems for cellulases expression has failed to provide both enzyme activity and yield. However, different systems and combinations should still be tested. Some studies have explored the use of surface-engineered cells that carry cellulases on their surfaces. Still, low activity and yields have been obtained (Garvey, et al., 2013).

Cellulosomes are a new, promising tool: they are large extracellular enzyme complexes that consist of a scaffolding protein and many bound enzymes. They are mainly produced by anaerobic bacteria, but there are evidences of cellulosomes produced by anaerobic fungi (Doi & Kosugi, 2004). An intriguing possibility for future studies might be the heterologous expression of cellulosomal genes to improve and refine the conversion of biomass and agricultural wastes to produce ethanol, amino acids and organic acids.

Acknowledgement

One of the author Dr Vibha Bhardwaj acknowledges ICGEB Trieste Italy for award of Arturo Falaschi SMART Fellowship and thanks to Director MINCyT for hosting ICGEB in the IBioBA Institute of Buenos Aires Argentina **.**

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Isabella Apruzzese. “Biogas Production From Organic Wastes Of Paper And Leather Industries.” IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB) , vol. 3, no. 4, 2017, pp. 08–14.